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(54) Title: SOMATOTROPIN MODIFICATIONS

(57) Abstract

Somatotropin modifications are described that have unexpected enhancements in conformational and chemical stability without loss of bioactivity. Alterations of these characteristics are obtained via modification of somatotropins by site-directed mutagenesis. These changes increase the storage stability of these proteins. Exemplary modifications include porcine somatotropins having histidine and arginine substitutions for glutamine and histidine at positions 19 and 20, respectively (Q19H, H20R), having glutamic acid substituted for phenylalanine at position 52 (F52E), and having glutamic acid substituted for leucine at position 137 (I137E). The modified somatotropins are useful to enhance growth characteristics in animal species, e.g., to increase the lean-to-fat ratio in pigs and milk production in cows.

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SOMATOTROPIN MODIFICATIONS

FIELD OF THE INVENTION

This invention provides novel single- or multiple-site substitution analogs of somatotropins that display enhanced stability relative to the unmodified protein.

BACKGROUND OF THE INVENTION

Somatotropins

Somatotropin (also referred to as St or growth hormone (GH)) was originally discovered in pituitary gland extracts from various animal species. These proteins are single chain molecules containing about 200 amino acids and two intramolecular disulfide bonds. Since these proteins contain no carbohydrate, lipid or other cofactors, their molecular weight is approximately 22,000 daltons. A number of mammalian, avian, and aquatic somatotropins have been well characterized with respect to their structure and biological behavior (see for example, J.L. Kostyo & A.E. Wilhemi, *Metabolism* 25:105-124 (1976); O.G.P. Isaksson, *et al.*, *Ann. Rev. Physiol.* 47:483-499 (1985); A.C. Paladini, *et al.*, *CRC Crit. Rev. Biochem.* 15:25-56 (1983)). Mammalian somatotropins are conserved molecules and similarities in amino acid sequences and protein structure are found between different species of animals.

Human and animal somatotropins, either extracted from pituitary gland tissue ("natural") or produced by recombinant organisms ("recombinant"), have important commercial applications. Human growth hormone enhances unusually slow growth in prepubescent children (Human Growth Hormone, S.Raiti, R.A. Tolman eds.; Plenum Press; New York (1986), Chapter 21). Bovine somatotropin, which enhances lactation in cows, is marketed for this purpose in some countries and is under review in the U.S. Both porcine (pSt) and bovine somatotropins (bSt) increase the percentage of lean tissue in the respective target animals with little impact on the feed to gain ratio. Neither protein has activity in humans (J.C. Juskevich & C.G. Guyer *Science* 249:875-884 (1990) and J.L. Kostyo & A.E. Wilhemi, *supra.*). It is inconvenient to administer these compounds to large numbers of farm animals on a daily basis, necessitating the development of sustained-release dosage forms for the administration of pSt and bSt. To ensure that the protein retains bioactivity during the delivery period, it must have high chemical and conformational stability. Previous efforts suggest that growth hormone for sustained delivery has lower stability than is needed for sustained delivery.

Disclosed herein are substitutions made in somatotropin that directly or indirectly result in stabilizing the protein. Each of these compounds is tested in the target species to determine if it is biologically active and retains commercial potential.

Structural Studies

Proteins contain amino acids linked by peptide bonds to form a linear chain. Frequently, the presence of multiple sulfhydryl-containing amino acid residues, i.e. cysteine

residues, permit oxidative cyclization of these compounds. Because of the large number of peptide bonds, and because many of the amino acid sidechains contain alcohol, acid, carboxamide, amine, guanidine, imidazole, indole, phenol, sulfide, disulfide, and mercaptan functional groups, there is strong potential for modification of the protein through the action of chemical or biological agents. In addition, it is well-known that the protein backbones of globular proteins are highly flexible. However, rather than move in solution as a random polymer, the protein typically adopts a semi-rigid tertiary structure. The ability of a protein to maintain this tertiary structure is referred to as conformational stability. Often, the latter structure is essential for maintaining biological activity. In these cases, loss of tertiary structure is a form of protein degradation. Further, chemical and conformational stability of a protein are interrelated. That is, reactions that modify the covalent structure of proteins can affect conformational stability, and structural changes that alter conformational stability can affect the rate of chemical degradation, often in an adverse manner.

A number of chemical and conformational degradation pathways have been identified for somatotropins. For example, deamidation, hydrolysis, and rearrangement reactions of the carboxamide sidechain of asparagine have been observed at positions 13, 47, 140, and 148 (C. Secchi, *et al., Int. J. Peptide Protein Res.* 28: 298 (1986), at positions 99 (International publication WO 87/01708), and position 129 (Wood, D.C., *et al., J. Biol. Chem.* 264:14741 (1989)), of the highly homologous animal somatotropins. Methionines at positions 5, 124, 149, and 179 have been shown to oxidize to the corresponding sulfoxide (O. Cascone, *et al., Int. J. Peptide Protein Res.* 16:299-305 (1980)). In addition, treatment of the protein with alkali under favorable conditions leads to intermolecular disulfide bridges generating reducible dimers. In somatotropin, these reactions appear to primarily involve cysteines of the short loop (positions 181 and 189). Conditions that lead to the loss of tertiary structure have also been defined. Denaturation of somatotropin occurs as a result of treatment with pH, guanidinium chloride, urea, and heat. In addition, partially denatured somatotropin has been shown to form a non-bioactive, poorly soluble aggregate species (D.N. Brems, *Biochemistry* 27:4541-4546 (1988)).

In order to minimize the occurrence of these degradation pathways, I have identified and synthesized modified forms of somatotropin that selectively stabilize the protein by minimizing individual degradation pathways while maintaining bioactivity in the target animal. These compounds are obtained by using site-directed modifications to generate nucleic acid substitutions in a somatotropin cDNA. The modified cDNA is then transformed into a host organism and the modified somatotropin analog expressed and purified. The term "site-directed" as used here is the process of controlled modifications of the primary structure of somatotropin. Single-site modifications are changes in the somatotropin gene sequence that produce one to three amino acid residue substitutions within a short segment of somatotropin. Single-site

substitution analogs are designed to address a specific degradation pathway (e.g., deamidation of asparagine at position 47). Multiple-site substitution analogs combine two or more single-site substitutions into somatotropin in order to further stabilize the protein. Site-directed modifications enhance St stability either by directly eliminating functional groups that undergo chemical degradation, or by reinforcing the conformational stability of native tertiary structure. In addition, it is possible to indirectly enhance chemical and conformational stability of the protein by increasing the solubility of the protein at neutral pH. This added solubility lessens the need to expose the protein to alkali or acid, thereby diminishing the presence of degradation products during administration.

Since the mechanism of action of these proteins is not clearly understood, the bioactivity of each somatotropin analog must be determined. In some cases, somatotropin variants showing diminished degradation do not retain the requisite levels of bioactivity needed in a commercial product. For example, single-site replacement of glutamic acid at position 56 with alanine significantly ($P < 0.05$) decreases the activity of this protein in a porcine growth bioassay as indicated by feed to gain ratios. Other laboratories have found that transgenic mice that produce a modified bSt containing aspartic acid at position 122 grow to the same size as normal mice. Transgenic mice containing arginine rather than glycine at position 119 of bSt possess a dwarf phenotype (W.Y. Chen *et al.*, *Molec. Endocrinol.* 5:1845-1852 (1991)). These results clearly indicate that the relationship between St primary structure and bioactivity is complex.

INFORMATION DISCLOSURE

A number of variant or modified somatotropins, also referred to as somatotropin analogs, are known. For example:

International publication WO 91/00870 discloses enhanced hydrophobicity, helical stability, and reduced aggregation of somatotropins by substitutions in the primary structure between residues 96 to 133.

International publication WO 90/08823 discloses the inhibition of intermolecular disulfide bond formation by replacing of C-terminal cysteine residues.

EP publication 0 355 460 and International publications WO 92/01789 and WO 90/02758 also report modified somatotropins by substitution of cysteine residues.

International publication WO 90/08164 discloses somatotropins displaying enhanced stability by replacement of the asparagine residue at position 99 of bovine somatotropin.

International publication WO 87/01708 discloses inhibition of hydrolytic effects, including deamidation, by replacement of the asparagine residue at position 99 of bovine somatotropin.

United States Patents 5,130,422 and 5,089,473 disclose somatotropins displaying enhanced stability by substitution of glutamine for asparagine located in the 95-101 region.

EP publication 0 488 279, which was published less than one year prior to the filing of this application, discloses somatotropin analogues with amino acid changes in the α-helix 3 and α-helix 2 regions of the molecule.

Ganter, C. and Pluckthun, A., *Biochemistry* 29:9395-9402 (1990) describe that substitutions of alanine for glycine can lead to changes in the stability of glyceraldehyde 3-phosphate dehydrogenase and that the influence of these substitutions is strongly dependent on amino acid position.

Hecht, M. H., *et al.*, *Proteins* 1:43-46 (1986) describe increased thermal stability of λ-Repressor protein by site-directed substitution of alanine for glycine in the α-helix region.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the results of accelerated stability studies of rpSt and modified rpSt analyzed by RP-HPLC. Together with data of Figures 2-8, this data shows that standard protein analysis is not sufficiently sensitive to permit the identification of specific stability enhancement. Peak area (as a percentage of initial peak area) is plotted as a function of time. Somatotropins are dissolved in sodium carbonate buffer, pH 9.8 and incubated at 30°C for up to 30 days for these analyses. rpSt (○); A48P (□); F103E (▾); C181,189S (▽); N99S (•). Further details are described in the Detailed Description section.

Figure 2 shows tryptic maps of native rpSt (top) and following 30 days of decomposition (bottom) in alkaline buffers. Peaks are identified using LC-MS (LC-MS: liquid chromatography-mass spectrometry). The numbers correspond to individual tryptic fragments of native rpSt and are identified in Table IV. Individual degradation products, labelled in Figure 2 (bottom) are identified in Table V. This approach therefore permits a more detailed and sensitive assessment of chemical stability.

Figure 3 shows a tryptic map of M5S following exposure to alkali buffers. This modified form of pSt is incubated as described in Figure 1. Samples are analyzed by tryptic mapping as described by J.J.Dougherty, et al. (*Analytical Biochemistry* 190:7-20 (1990)). In brief, this procedure calls for the incubation of somatotropin with trypsin at pH 8.3 at 5°C for 24 hours. The digests are mapped by HPLC. Additional details are described in the Detailed Description section.

Figure 4 shows a tryptic map of N47D following exposure to aqueous alkaline. Incubation and analyses are briefly described in the legends of Figures 1 and 3, respectively.

Figure 5 shows a tryptic map of A48P following exposure to alkaline buffers. Incubation and analyses are briefly described in the legends of Figures 1 and 3, respectively.

Figure 6 shows a tryptic map of N99S following exposure to alkaline buffers. Incubation and analyses are briefly described in the legends of Figures 1 and 3, respectively.

Figure 7 shows a tryptic map of C181,189S following exposure to alkaline buffers.

Incubation and analyses are briefly described in the legends of Figures 1 and 3, respectively.

SUMMARY OF THE INVENTION

This invention relates to modified somatotropins, or somatotropin analogs, having one or more amino acid changes that enhance the chemical and conformational stability of the protein while maintaining levels of bioactivity which are the same as, or similar to, the native protein.

A first aspect of the invention relates to somatotropin analogs wherein one of the following single-site substitutions is made in the protein: serine replacing methionine at amino acid position 5 (the analog designated M5S), serine replacing asparagine at position 13 (N13S), leucine for the valine found at amino acid position 15 (V15L), histidine replacing glutamine at position 19, and arginine replacing histidine at position 20 (Q19H,H20R), glutamic acid replacing glycine at position 40 (G40E), aspartic acid replacing asparagine at position 47 (N47D), proline replacing alanine at position 48 (A48P), glutamic acid replacing phenylalanine at position 52 (F52E), asparagine replacing glycine at position 63 (G63N), phenylalanine replacing tryptophan at position 86 (W86F), glutamic acid replacing glycine at position 88 (G88E), alanine replacing threonine at position 98, serine replacing asparagine at position 99, and alanine replacing serine at position 100 (T98A,N99S,S100A), glutamic acid replacing phenylalanine at position 103 (F103E), leucine replacing methionine at position 124 (M124L), glutamic acid replacing isoleucine at position 137 (I137E), serine replacing glutamine at position 140 (Q140S), serine replacing asparagine at position 148 (N148S), or leucine replacing methionine at position 179 (M179L).

More particularly, the invention relates to analogs of porcine or bovine somatotropin, most particularly porcine, having one of the substitutions listed above.

A second aspect of the invention relates to multiple-site somatotropin analogs wherein two or more of the following single-site substitutions is made in the protein: serine replacing methionine at amino acid position 5 (M5S), serine replacing asparagine at position 13 (N13S), leucine replacing valine at position 15 (V15L), histidine replacing glutamine at position 19, and arginine replacing histidine at position 20 (Q19H,H20R), glutamic acid replacing glycine at position 40 (G40E), aspartic acid replacing asparagine at position 47 (N47D), proline replacing alanine at position 48 (A48P), glutamic acid replacing phenylalanine at position 52 (F52E), asparagine replacing glycine at position 63 (G63N), phenylalanine replacing tryptophan at position 86 (W86F), glutamic acid replacing glycine at position 88 (G88E), alanine replacing threonine at position 98, serine replacing asparagine at position 99, and alanine replacing serine at position 100 (T98A,N99S,S100A), serine replacing asparagine at position 99 (N99S), glutamic acid replacing phenylalanine at position 103 (F103E), leucine replacing methionine at position 124 (M124L), serine replacing asparagine at position 125 (R125S), glutamic acid replacing isoleucine at position 137 (I137E), serine replacing glutamine at position 140 (Q140S),

serine replacing asparagine at position 148 (N148S), leucine replacing methionine at position 179 (M179L), or serine replacing cysteine at positions 181 and 189 (C181,189S).

Particularly, this aspect of the invention relates to somatotropin analogs wherein the following multiple-site substitutions are made in the somatotropin protein:

(a) V15L, A48P, F52E;

(b) A48P, N99S, CI8I,189S;

(c) F52E, G88E, F103E, I137E;

(d) V15L, Q19H,H20R, A48P, F52E;

(e) N13S, W86F, Q140S, N148S, M124L, M179L; and

(f) V15L, A48P, F52E, N99S, F103E, C181,189S.

More particularly, the invention relates to analogs of porcine or bovine somatotropin, most particularly porcine, having the multiple-site substitutions listed above.

An additional aspect of the invention also relates to expression plasmids comprising cDNA encoding one of the somatotropin analogs of the invention.

A third aspect of the invention relates to host cells transformed with expression plasmids comprising cDNA encoding a porcine or bovine somatotropin of the invention.

Additional aspects of the invention relate to a method of increasing the growth of an animal, a method for increasing milk production in a cow, and a method for increasing lean-to-fat ratio in a pig, each of the methods of the invention comprising administering to the animal an effective amount of a somatotropin analog having single-site or multiple-site amino acid substitutions as described above.

In particular, one method of the invention comprises administering to an animal an effective amount of a somatotropin having one of the following single-site substitutions in the amino acid sequence of the protein: serine replacing methionine at amino acid position 5 (the analog designated M5S), serine replacing asparagine at position 13 (N13S), leucine for the valine found at amino acid position 15 (V15L), histidine replacing glutamine at position 19, and arginine replacing histidine at position 20 (Q19H,H20R), glutamic acid replacing glycine at position 40 (G40E), aspartic acid replacing asparagine at position 47 (N47D), proline replacing alanine at position 48 (A48P), glutamic acid replacing phenylalanine at position 52 (F52E), asparagine replacing glycine at position 63 (G63N), phenylalanine replacing tryptophan at position 86 (W86F), glutamic acid replacing glycine at position 88 (G88E), alanine replacing threonine at position 98, serine replacing asparagine at position 99, and alanine replacing serine at position 100 (T98A,N99S,S100A), glutamic acid replacing phenylalanine at position 103 (F103E), leucine replacing methionine at position 124 (M124L), glutamic acid replacing isoleucine at position 137 (I137E), serine replacing glutamine at position 140 (Q140S), serine replacing asparagine at position 148 (N148S), or leucine replacing methionine at position 179

(M179L).

An additional method of the invention comprises administering to the animal an effective amount of a multiple-site somatotropin having two or more of the following: serine replacing methionine at amino acid position 5 (designated M5S), serine replacing asparagine at position 13 (N13S), leucine replacing valine at position 15 (V15L), histidine replacing glutamine at position 19, and arginine replacing histidine at position 20 (Q19H,H20R), glutamic acid replacing glycine at position 40 (G40E), aspartic acid replacing asparagine at position 47 (N47D), proline replacing alanine at position 48 (A48P), glutamic acid replacing phenylalanine at position 52 (F52E), asparagine replacing glycine at position 63 (G63N), phenylalanine replacing tryptophan at position 86 (W86F), glutamic acid replacing glycine at position 88 (G88E), serine replacing asparagine at position 99 (N99S), alanine replacing threonine at position 98, serine replacing asparagine at position 99, and alanine replacing serine at position 100 (T98A,N99S,S100A), glutamic acid replacing phenylalanine at position 103 (F103E), leucine replacing methionine at position 124 (M124L), serine replacing asparagine at position 125 (R125S), glutamic acid replacing isoleucine at position 137 (I137E), serine replacing glutamine at position 140 (Q140S), serine replacing asparagine at position 148 (N148S), leucine replacing methionine at position 179 (M179L), or serine replacing cysteine at positions 181 and 189 (C181,189S).

More particularly, this aspect of the method of the invention comprises administering to the animal an effective amount of a somatotropin having multiple-site substitutions selected from the group consisting of (a) V15L, A48P, F52E; (b) A48P, N99S, CI8I,189S; (c) F52E, G88E, F103E, I137E; (d) V15L, Q19H,H20R, A48P, F52E; (e) N13S, W86F, Q140S, N148S, M124L, M179L; and (f) V15L, A48P, F52E, N99S, F103E, C181,189S.

DETAILED DESCRIPTION

The present invention discloses somatotropin modifications that display enhanced conformational and chemical stability as compared to native pituitary or recombinantly derived somatotropins. Single site modifications meeting this criteria include, for example, M5S, V15L, Q19H,H20R, A48P, G40E, N47D, F52E, G88E, F103E, I137E, and R125S. Additional modifications meeting this criteria and included as multiple-site analogs, include, for example, N99S, C181,189S. This shorthand notation shows, for example, that in M5S, methionine at position 5 of the rpSt primary structure, is replaced by serine.

Sequence ID:1 shows the primary structure of pSt as isolated from pigs. Other closely related somatotropin molecules having pSt activity have also been identified due to genetic polymorphism. Because of these changes and the slightly altered structure of homologous somatotropins, minor changes in the numbering and specific residues in the primary structure are found in the literature.

The identity of specific protein modifications that enhance somatotropin stability have been made by characterizing single-site and multiple-site amino acid substitution somatotropins in chemical and bioactivity studies.

The strategies used to enhance the overall stability of the protein include: (i) modifications of somatotropin primary structure to enhance conformational stability of the protein, and/or (ii) specific elimination of somatotropin residues that are known to be involved in the decomposition of the protein, and/or (iii) modifications of somatotropin primary structure that permit the protein to be handled in neutral aqueous media. This strategy can also result in an indirect stabilization of protein since it can now be processed, stored, and formulated under conditions that will have a much lower propensity for decomposing somatotropins.

The following list details amino acid residue replacements that are expected to enhance the stability of the protein according to one of the three principles described above:

Replacing methionine at position 5. Suitable amino acid substitutions which will remove this decomposition site and enhance solubility of the protein are serine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, proline, and threonine.

Replacing asparagine at position 13. Suitable amino acid substitutions which will remove this hydrolytic degradation site are serine, glutamic acid, threonine, glutamine, aspartic acid, lysine, arginine, alanine, and histidine.

Replacing valine at position 15. Valine and isoleucine will reinforce the conformational stability of somatotropin at this position.

Replacing glutamine at position 19 with histidine and replacing histidine at position 20 with arginine will alter this degradation site.

Replacing glycine at position 40. Suitable amino acid substitutions which will remove the conformational flexibility of the backbone include tyrosine, proline, threonine, serine, glutamic acid, asparagine, glutamine, aspartic acid, lysine, and arginine.

Replacing asparagine at position 47. Aspartic acid and glutamine are suitable to diminish hydrolytic degradation at this position.

Replacing alanine at position 48 with proline will enhance the conformational stability of the backbone of the molecule.

Replacing phenylalanine at position 52. Suitable amino acid substitutions which will enhance the aqueous solubility of the protein are glutamic acid, alanine, glycine, tyrosine, proline, threonine, serine, histidine, asparagine, glutamine, aspartic acid, lysine, and arginine.

Replacing glycine at position 63. Suitable amino acids substitutions which will enhance the conformational stability of the protein backbone include asparagine,

arginine, lysine, aspartic acid, glutamine, glutamic acid, histidine, serine, threonine, proline, tyrosine, and alanine.

Replacing tryptophan at position 86. Suitable amino acids substitutions which will remove this degradation site without substantively enhancing the hydrophilicity of the protein interior include phenylalanine, tyrosine, leucine, isoleucine, and valine.

Replacing glycine at position 88. Glutamic acid and aspartic acid will reinforce the conformational stability of the protein at this position.

Replacing threonine at position 98. Suitable amino acids which are expected to elimante secondary decomposition reactions near residue 99 while maintaining overall solubility of the protein include alanine, proline, glycine, glutamic acid, aspartic acid, lysine, and arginine.

Replacing asparagine at position 99 with serine is known to enhance the stability of the molecule.

Replacing serine at position 100. Suitable amino acids which are expected to elimante secondary decomposition reactions near residue 99 while maintaining overall solubility of the protein include alanine, proline, glycine, glutamic acid, aspartic acid, lysine, and arginine.

Replacing phenylalanine at position 103. Suitable amino acid substitutions which will enhance the aqueous solubility of the protein include glutamic acid, alanine, glycine, tyrosine, proline, threonine, serine, histidine, asparagine, glutamine, asparatic acid, lysine, and arginine.

Replacing methionine at position 124. Suitable amino acids which will eliminate the oxidative degradation of this residue include leucine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, proline, serine, and threonine.

Replacing arginine at position 125 with serine is also known to enhance the stability of the molecule.

Replacing isoleucine at position 137. Suitable amino acid substitutions which will enhance the aqueous solubility of rpSt include glutamic acid, alanine, glycine, tyrosine, proline, threonine, serine, histidine, asparagine, glutamine, aspartic acid, lysine, and arginine.

Replacing glutamine at position 140. Suitable amino acid substitutions which will remove deamidation at this site are serine, alanine, aspartic acid, lysine, arginine, histidine, glutamic acid, and threonine.

Replacing asparagine at position 148. Suitable amino acid substitutions which will remove deamidation at this site are serine, alanine, aspartic acid, lysine, arginine, histidine, glutamic acid, glutamine, and threonine.

Replacing methionine at position 179. Suitable amino acids which will eliminate the oxidative degradation of this residue include leucine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, proline, serine, and threonine.

Replacing cysteine residues at positions 181 and 189 with serine is also known to enhance the stability of the molecule.

The strategy outlined above can be used by those skilled in the art to readily and easily characterize additional modified somatotropins of the invention. As these studies focus on increased chemical and conformational stability of somatotropins, while maintaining bioactivity in a target animal, the modified somatotropins of the invention are first characterized by means of the chemical and conformational studies described in more detail below. Thus, to be a candidate for *in vivo* bioassays, a somatotropin generally exhibits an enhancement (as compared to the native somatotropin) in one of the stability or conformational studies described herein. It is contemplated that some degree of decreased bioactivity is acceptable due, in part, to the advantage gained by enhanced stability. Preferred modifications enhance both chemical and/or conformational stability as well as bioactivity in the host species.

Synthesis of Modified Somatotropins

These compounds are synthesized via selective site-directed mutagenesis, using cDNAs encoding replacements of the native amino acid residues. Site-directed mutagenesis is a technique well known in the art of recombinant technology.

Several techniques for site-directed mutagenesis have been developed for introducing specific changes in a DNA sequence and may be used to produce the compounds of the instant invention (Kramer, E.,W, *et al.*, *Nucl. Acids Res.* 12:9441-56 (1984); Mandecki, W., *PNAS USA*, 83:7177-81 (1986); Zollar, M.J. and Smith, M., *Nucl. Acids Res.* 10:6487-6500 (1982); Norrander, J., *et al.*, *Gene* 26:101-106 (1983); Kunkel, T.A., *PNAS USA* 82:488-92 (1985); Schold, A., *et al.*, *DNA* 3:469-77 (1984); and Marotti, K.M. and Tomich C-S.C., *Gene Anal. Tech.* 6:67-70 (1989). The primer-directed mutagenesis technique of Marotti and Tomich, using only one primer for the mutagenesis reaction and gene 32 protein to increase mutagenesis efficiency is either performed as described by the authors or performed with slight variations. One of the variations is to use a longer mutagenic oligonucleotide (about 40 bases) in the mutagenesis reaction and to screen candidates using a shorter oligonucleotide (<20 bases). The other variation in the protocol is to transform the mutagenic reaction into either BHM71-18 mutS::Tn10 or BST-1c, pool the colonies by scraping the colonies together, isolating vector DNA and re-transforming into either BST-1c or MC1061. Electroporation is used when the initial transformation yields are low. Procedures leading to the MC1061 and BST-1c culture systems are described detail in International publication W088/06186. BST-1C is available from the Agricultural Research Service Culture Collection in the Northern Regional Research Center,

Peoria, IL, accession number NRRL B-18303. MC1061 is purchased from Clontech Laboratories, Inc., Palo Alto, CA. BHM71-18 mutS::Tn10 is purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN, as a part of a Site-Directed Mutagenesis Kit (Cat. No. 1027-492).

It is apparent to those skilled in the art that due to the degeneracy of the genetic code that other triplets (coding for the same amino acids) may be substituted in the primers used to make the modified somatotropins of the invention. It is also apparent to those skilled in the art that by following the teachings of the present disclosure additional amino acid residues, having chemical and/or physical characteristics similar to those described above and substituted at the amino acid positions described above, may be easily substituted into a somatotropin molecule in order to obtain the same or similar biophysical and/or biological enhancements. For example, although methionine at position 5 is substituted by serine within this invention, other amino acids with chemical structures that resemble serine (e.g. threonine, alanine etc.) will produce similar advantages without perturbing the protein tertiary structure.

It is understood by those skilled in the art that somatotropins identified from different species are essentially homologous in amino acid sequence and physical structure. Although the sequence changes described in the Examples have been made within pSt, the techniques are equally applicable to any somatotropin, especially mammalian somatotropins, having the requisite amino acid residues available for conversion. The amino acid sequence and/or nucleic acid sequence of species other than porcine are well known to those skilled in the art.

The porcine somatotropin gene (PSt1) used in these studies is isolated from a porcine pituitary cDNA library constructed at the Upjohn Company and is described in International publication WO 88/06186. The pURA plasmid, used for expression of the pSt gene, is described in International publication WO 88/06186. The vector is modified to contain the *Serratia marcescens* trp promoter as described in WO 90/05186 and the ConSD ribosome-binding site as described by Curry and Tomich *supra*. The *E. coli* strain and preferred host for expression of the somatotropin analogs of the invention is BST-1c.

Transformed cells containing the modified porcine gene are grown at the 10 liter scale. After fermentation the cell are centrifuged, and the modified porcine somatotropins are isolated using a bench scale modification of procedures described International publication WO 87/00204.

Once isolated, the structure of the somatotropin analog is verified by use of a quantitative tryptic map (J.J. Dougherty, *et al.*, *Analytical Biochemistry* 190:7-20 (1990)). The trypsin used in these studies is previously treated with TPCK (1-tosylamide-2-phenylethyl chloromethyl ketone) in order to irreversibly inhibit chymotrypsin that may contaminate the product (Worthington Biochemical, Freehold, NJ.). This enzyme is further purified via RP-

HPLC chromatography. Briefly, formation of the digests calls for incubating somatotropin (2 mg) dissolved in 1 mM NaOH (7 ml), and 1 M Tris, 10 mM $CaCl_2$, pH 8.3 (1 ml) at 5°C. The reaction mixture is incubated at 5°C for 24 hours and quenched with 0.1 ml of 10% trifluoroacetic acid. Tryptic digests, prepared in this manner, are chromatographed on the HPLC system described above using a C8 column (4.6mm ID x 25cm, 5 µm, 300 Å pore; Regis Chem., Morton Grove, IL). Buffers for chromatography are: A - 0.1% TFA in water, B - 70% CH_3CN/0.07%TFA. Following injection, the column is eluted for 5 minutes in 1% B and chromatographed using a gradient of 1% B to 64.3% B in 120 minutes. Flow rate is 1.2 ml/min. The column effluent is monitored at 214 nm with column temperature at 40°C. Because of the reproducibility of the elution profile and the resolving capacity of the HPLC analysis, it is generally easy to separate and isolate any tryptic fragments that differ in retention time from those derived from wild-type somatotropins. The peptide sequence of this region can be confirmed either by mass spectrometry or by NH_2-terminal sequence analysis. Protein purity is determined using RP-and SEC-HPLC, and isoelectric focusing. These techniques are well known by those skilled in the art.

Somatotropin concentrations are determined by the absorbance at 278 nm using an extinction coefficient of 15,270 M^{-1} (Burger, H.G., *et al., J. Biol. Chem.* 241:449-457 (1966)).

Determination of Relative Conformational Stability

Demonstration that these site-directed modifications enhance the conformational stability of the protein is determined by the comparison of equilibrium denaturation curves as previously described (Havel, H.A., *et al., Biochemistry* 25:6533-6538 (1986); Brems, D.N., *et al., Biochemistry* 25:6539-6543 (1986); Brems, D.N., *et al., J. Biol. Chem.* 262:2590-2596 (1987); Brems, D.N., *et al., Biochemistry*, 24:7662-7668 (1985)). The buffer used in equilibrium denaturation analysis is a stock solution of 50 mM NH_4HCO_3, pH 8.5. Subsequent sample dilutions to the appropriate concentrations of protein and denaturant are made using the stock solution buffer and 8.0 M guanidine HCl. The stock denaturant and buffer solutions are filtered through 0.45 µm disposable filters (Corning Inc., Corning, N.Y.) prior to use. Both the stock solutions and prepared samples are stored at 4°C. Before spectral analysis, samples are allowed to equilibrate at ambient temperature for several hours. Protein concentrations are determined using a dye-binding method (bicinchoninic acid). This method is described by Smith, P. et al., Anal. Biochem.

Fluorescence measurements are performed on a photon-counting spectrofluorimeter (SLM Instruments, Inc., Urbana, IL). Spectra are obtained after excitation at 295 nm by monitoring protein emission at 355 nm. 2nd derivative UV absorption measurements are performed on scanning (Aviv Associates, Lakewood, N.J.) and diode-array spectrometers (Hewlett Packard Scientific Instruments, Palo Alto, CA). Absorption spectra are obtained

between 280 and 300 nm. Subsequent data handling is performed using algorithms supplied with each vendor's software.

Circular dichroic (CD) measurements are performed on (JASCO 500C and 700 series spectropolarimeters (Japan Spectroscopic Company, Tokyo, Japan). Near UV (275 to 325 nm) and far UV (226 to 218 nm) data are obtained for analysis. Raw data from this technique, obtained in units of ellipticity (millidegrees), is expressed in units of mean residue ellipticity (deg•cm^2/dmol) using the equation:

$$[\theta] = (\theta_{obs} \bullet MRW)/(100 \bullet l \bullet c)$$

(Where θ_{obs} and $[\theta]$ are the ellipticity and mean residue ellipticity, MRW is the mean residue weight (115 in units of g/mole•residue), l is the path length in decimeters, and c is the protein concentration in grams/milliliter.)

Non-linear least-squares and other analyses of the data from each of the techniques discussed above are performed using Sigmaplot 4.1 (Jandel Scientific, Corte Madera, CA). Analysis of the equilibrium data from each technique is based on the two state mechanism for protein folding:

$$\text{Native (folded)} \leftrightarrow \text{Denatured (unfolded)}$$

where spectroscopic measurements of an observable parameter at discrete concentrations of denaturant result in a sigmoidal denaturation curve. This analysis, described by Pace, C.N. (Methods in Enzymology, 131:266-280 (1986)) assumes the transition from folded to unfolded states does not contain intermediates in appreciable concentrations.

Determination of Relative Chemical Stability

Somatotropins (2 mg/ml) are dissolved in 0.05M sodium carbonate, pH 9.8. The vials are capped, placed in an oven and incubated at 30°C for up to 30 days. Daily aliquots are taken and stored at -20°C prior to analysis.

Aliquots (20 µl) are analyzed using twin pump reverse phase-HPLC with UV detection (Millipore, Milford,MA) on a 4.6mm ID x 25cm C_4 column (The Sep/ara/tions Group, Hesperia,CA). Gradient conditions are 40%-70% CH_3CN/0.1% TFA, 1% minute^{-1} with flow rate set at 1 ml/min. Column effluent is monitored at 214 nm. The temperature of the column is 25°C.

The ability of RP-HPLC to discern differences in chemical stability relies on the ability of the chromatography system to resolve all impurities from the starting material. Therefore, inability to detect enhancements in chemical stability by this technique is not proof that the compounds have greater stability. Because it provides more detailed molecular characterization, tryptic mapping in combination with mass spectral analysis of individual fragments is more reliable. Tryptic mapping is performed as described above.

Determination of Relative Solubility

Proteins are dissolved in 0.05M sodium carbonate pH 9.8, at a protein concentration of 50mg/ml. The pH is adjusted with 50% NaOH where necessary. After filtration, a 250ul aliquot is withdrawn for dialysis. Microdialysis is conducted in a low volume chamber, fitted with a dialysis membrane (6000-8000 molecular weight cutoff). The unit is connected to a reservoir containing 1 liter of 0.05M sodium carbonate pH 9.8. In addition, a second unit is attached to a reservoir containing 0.04M sodium phosphate pH 7.0. A Minipuls 2 peristaltic pump (Gilson, Middleton, WI) is used to pump buffer through the dialysis units at a flow rate of 30ml/hr. Samples are dialyzed for 20 hours at 5°C. The dialyzed samples as well as the starting material are then centrifuged at 13,000 rpm for 6 minutes in an Eppendorf 5415 centrifuge (Brinkman Instruments, Des Plaines, IL). The protein concentration of the supernatant liquid is determined using the BCA assay (Pierce Chemical Company, Rockford, IL; P.K. Smith, *et al.*, *Anal. Biochem.* 150:76-85 (1985)).

Determination of Relative Bioactivity

The compounds of the invention may be tested for bioactivity by measuring growth in hypophysectomized rats and the target species following repeated administration.

The hypophysectomized rat growth assay is generally recognized by those skilled in the art as an appropriate method for obtaining a relatively rapid measure of the relative potency of somatotropins. The modified somatotropins of the present invention are screened using hypophysectomized rats following the procedure of Evans, H.M. and Long J.A., *Anat. Rec.* 21:61 (1921), and M.D. Groesbeck and A.F. Parlow, *Endocrinology* 120:2582-2590 (1987), as modified below. Briefly, linearity of the assay is determined via the subcutaneously injection of eight hypophysectomized rats with somatotropin at doses of 2, 10, 50, and 250 µg/rat-day. The modified somatotropins are tested at 15 and 60 µg/ml. The weight of the rats are measured daily on the first day and up to the day following the final injection. Relative increases in total body weight are recorded using various modified somatotropins of the invention with the native form of the molecule, either natural somatotropin and/or recombinant somatotropin, serving as a positive control.

While relative biopotency of each analog is determined in the rat growth assay, activity in the target species is the primary determinant of commercial potential for these somatotropins. For most animal species, assays are known in the art which are appropriate and sensitive for determining the relative bioactivity of the compounds. In addition, one skilled in the art can readily design assays for a target species. For instance, the bioactivity of the modified somatotropins of the invention can be measured using the swine growth assay using Yorkshire pigs. In this procedure, 48 gilts and barrows are sorted according to weight, and randomly assigned to four blocks of 6 pens each. Each pen contained 2 gilts and 2 barrows. Treatment was assigned randomly to pens within a block. Following this exercise, the animals are left for

a 7 day acclimation. They are then injected with somatotropin or modified somatotropin at a rate of 60 µg/kg/d for 14 days. Positive controls are administered recombinant rpSt. The pretreatment weight is compared with posttreatment weight with measurements at days 1, 15, and 22 of the experiment.

Use of the Compounds of the Invention

The compounds of the invention can be used to enhance growth characteristics in the species in which the native somatotropin has been shown to have growth-related bioactivity. In addition, because growth hormones are very similar in their amino acid sequences, hormones originating from one species may enhance the growth of other animal species that evolved at earlier times. Where appropriate, the compounds of the invention are suitable for use in this manner as well. Generally, somatotropins of less highly evolved species, such as cattle and pigs, have not been found to be functional in more highly evolved species, such as humans.

Administration of the compounds of the invention into animals is done according to known methods using any route effective to deliver the required dosage to the animal's circulatory system. Modes of administration include those typically encountered for the species of choice. Because proteins in general are susceptible to degradation in the digestive system, injection is preferred via an intramuscular or subcutaneous route. The use of sustained or prolonged release formulations or implants are also suitable modes. Generally, injection of a sustained release formulation is preferred.

The effective dosage range depends on the animal, as well as the age, weight, and general health of the animal to be treated. These and other parameters which are needed to determine the effective dosage range for a given species is well within the purview of one skilled in the art. For instance, in bovine the effective amount is in the range of 1.0 to 200 milligrams per animal per day. In pigs, for instance the effective amount is about 60 µg/kg/d.

The potential commercial use of the compounds of the invention in non-human animals derives from their enhancement of the animal's metabolism. Thus, administration of an effective amount of the porcine analogs of the invention allows a pig to grow leaner in a shorter time span and requires less feed to do so. In addition, administration of an effective amount of the bovine analogs of the invention allows milk producing animals, such as dairy cattle, to produce more milk for the same or lower amount of feed.

Definitions

The compounds of the invention are identified using a letter-number-letter string which designates the location of the substitution and the native and substituted amino acid. The letters of this string follow the accepted single letter designation for amino acids. The single letter designation and the amino acid to which it corresponds are shown in Chart 1. Using this nomenclature, the first letter (X--) designates the native amino acid found in wild-type

somatotropin; the second letter (--X) designates the amino acid that is substituted in the analog. The number (-0-) represents the sequential location of the amino acid in the native porcine somatotropin. Thus, V15L designates a somatotropin analog of the invention where leucine is substituted for the valine residue found at position 15 of native porcine somatotropin, and A48P designates the somatotropin analog of the invention having proline substituted for alanine at position 48 of the native porcine somatotropin.

"Animal" means both human and nonhuman animals.

An "effective amount" is the amount of a modified somatotropin of the invention which results in the desired biological effect in the target animal. "Effective amount" includes the dosage level which is efficacious as well as the period of time such a dose is administered to attain or maintain the efficacious dose.

"Microorganism" means both single cellular prokaryotic and eukaryotic organisms such as bacteria, yeast, actinomycetes and single cells from higher plants and animals grown in cell culture.

"Single-site" means single or multiple changes in the somatotropin gene sequence which result in a one to three amino acid residue substitution within a short segment of the somatotropin protein. Thus, for example, the analogs A48P and Q19H,H20R designate single-site substitutions.

"Multiple-site" substitutions or analogs combine two or more single-site substitutions into an individual protein in order to further enhance desirable characteristics. Following this nomenclature, the analog V15L, A48P, F52E, N99S, F103E, C181,189S designates a multiple-site modified somatotropin.

"Native" somatotropin refers to somatotropins having amino acid sequences which are the same as the naturally-occurring forms of the molecule. Native somatotropins may be derived from either natural or recombinant sources. Due to the molecular heterogeneity of somatotropins, the position numbers of amino acid residues of the various somatotropins may differ. The term native somatotropin includes these naturally occurring species. Sequence ID:1 illustrates one species of porcine somatotropin (pSt). The numbering for other somatotropins may differ. However, using the amino acids numbered 15, 48, 52, 88, 99, and 125 in Sequence ID:1, those of ordinary skill in the art can readily locate corresponding amino acids in other native somatotropins, or modified forms of these somatotropins, to achieve the desired enhanced conformational and chemical stability of the somatotropins of the invention.

"Somatotropin" or "St" means an animal somatotropin and includes somatotropins derived from either the pituitary gland tissue ("natural somatotropin") or from microorganisms transformed by recombinant genetics to produce either naturally-occurring or modified forms of somatotropin ("recombinant somatotropin"). When a specific mammalian source is named (such

as a somatotropin of porcine origin (porcine somatotropin or pSt)), the somatotropin includes those derived from either pituitary sources or from transformed microorganisms.

Many of the methods employed to produce the novel compounds of the invention and the various embodiments thereof involve standard laboratory techniques relating to recombinant DNA engineering. Such techniques are known by those skilled in the art or are readily obtained by reference to standard laboratory texts. Examples of such texts are: J. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972); D. A. Morrison, "Transformation and Preservation of Competent Bacterial Cells by Freezing", *Methods Enzymol.* 68:326-331 (1979); and Sambrook, J., *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989). Except where noted, all chemicals and materials employed, or their equivalents, are readily available from commercial vendors.

The compounds of the present invention, and their use, may be more fully understood by way of the following embodiments. These embodiments should in no way be construed to be limiting of the specification and claims. In the foregoing specification and examples which follow, the entire contents of all cited references are incorporated by reference.

Examples 1-14: Single-site modified somatotropins

Example 1: Production of M5S.

A site-directed mutagenic technique for double-stranded primer extension is used to introduce altered codons for methionine at amino acid position 5 in the PSt1 cDNA gene. In this method, the target sequence is cloned into a suitable plasmid and plasmid DNA is prepared. The plasmid DNA is denatured by treatment with NaOH which causes "nicks" in the DNA molecule deoxyribose-phosphate backbone. This relaxes the DNA and permits an oligomer containing the desired sequence changes to hybridize to the target site. The 3' end of the oligomer generates a primer for the DNA polymerase activity of the Polymerase A Klenow fragment which extends the primer, synthesizes a new DNA strand containing the mutagenic oligomer and displaces the normal complementary strand. The extension reaction increases the probability of the incorporation by genetic recombination. The DNA is transformed into competent cells and the resultant colonies are screened by colony filter hybridization. Plasmid DNA is isolated and sequenced from positive candidates.

The oligomers used to construct the position 5 serine change in the Pst1 gene are produced by techniques described in International publication WO 88/06186 and is shown in Sequence ID:2. An oligonucleotide so produced and set forth above contains the proper change.

Example 2: Production of V15L

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:3), a somatotropin analog having leucine at position 15 is also constructed.

Example 3: Production of G40E

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:4), a somatotropin analog having glutamic acid at position 40 is also constructed.

Example 4: Production of N47D

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:5), a somatotropin analog having aspartic acid at position 47 is also constructed.

Example 5: Production of A48P

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:6), a somatotropin analog having proline at position 48 is also constructed.

Example 6: Production of F52E

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:7), a somatotropin analog having glutamic acid at position 52 is also constructed.

Example 7: Production of E56A

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:8), a somatotropin analog having alanine at position 56 is also constructed.

Example 8: Production of G88E

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:9), a somatotropin analog having glutamic acid at position 88 is also constructed.

Example 9: Production of N99S

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:10), a somatotropin analog having serine at position 99 is also constructed. This substitution has also been described in International publication WO 90/00870.

Example 10: Production of F103E

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:11), a somatotropin analog having glutamic acid at position 103 is also constructed.

Example 11: Production of R125S

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:12), a somatotropin analog having

serine at position 125 is also constructed.

Example 12: Production of I137E

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:13), a somatotropin analog having glutamic acid at position 137 is also constructed.

Example 13: Production of Q19H,H20R

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:14), a somatotropin analog having histidine at position 19 and an arginine at position 20 is also constructed.

Example 14: Production of C181,189S

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids, a somatotropin analog having serines at position 181 and 189 is also constructed. This substitution has also been described in International publication WO 90/08823.

Example 15: Production of G63N

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:15), a somatotropin analog having asparagine at position 63 is also constructed.

Example 16: Production of T98A,N99S,S100A

Following the techniques of Example 1, but substituting the appropriate oligomers encoding the desired amino acids (as seen in Sequence ID:16), a somatotropin analog having alanine at position 98, serine at position 99, and alanine at position 100 is also constructed.

Examples 17-22: Multiple-site modified somatotropins

Multiple-site modified somatotropins can be made using the single-site mutagenic methods described above, each cycle of which is repeated for the addition of each single-site substitution. In this way modified somatotropins incorporating any number of single-site modifications can be made.

Alternatively and preferably, large DNA sequences can be constructed by combining long oligonucleotide synthesis with Polymerase Chain Reaction (PCR) as has been demonstrated by R.W. Barnett and H. Erfle, *Nucleic Acid Research* 18:3094 (1990), P.J. Dillon and C.A. Rosen, *Biotechniques* 9:298-299 (1990), and R.M. Horton, *et al.*, *Gene* 77:61-68 (1990). An entire gene of porcine somatotropin can be constructed containing the desired codon changes using this approach. Two long oligonucleotides of about 200 bases is synthesized as described below (see, "Synthesis of Oligonucleotides"). The concentration of the full length oligonucleotides is low due to the inefficiency in the addition of each nucleotide. These two oligonucleotides are designed to overlap by 20 to 30 bases, and when mixed together can be

extended by a DNA polymerase to form a larger 400 base pair fragment. This fragment can be amplified using PCR. The fragment is then isolated, cut with restriction enzymes and cloned into an appropriate expression vector. By generating two or three sets of these fragments an entire porcine somatotropin gene can be constructed.

Synthesis of Oligonucleotides

Oligonucleotides are made by the phosphoramidite method for DNA synthesis. In brief, this process uses as the starting material the 5' dimethoxytrityl ether of a nucleoside bound to a silica support. This nucleoside will be the 3'-OH end of the product oligonucleotide. The first step is detritylation with acid. Then the nucleotide phosphoramidite to be added is activated. The addition step occurs in less than 3 minutes and is 95 to 97% complete. Any chains which did not undergo addition is capped by acetylation so that it will not react in subsequent steps. Next, the internucleotide linkage is then converted from the phosphite to the more stable phosphate by oxidation. This compound is detritylated and the cycle continues until the last nucleoside is added. At this point, the oligonucleotide is the bases A, G, and C. The chain is then cleaved from the support with ammonium hydroxide. After, the solution containing the DNA is removed from the instrument, the protecting groups are cleaved by a 8-hour treatment with ammonium hydroxide at 55°C.

Example 17: Production of V15L, A48P, F52E, N99S, F103E, C181,189S

Following the techniques described above, a somatotropin multiple-site analog having leucine at position 15, proline at position 48, glutamic acid at position 52, serine at position 99, glutamic acid at position 103, and serine residues at positions 181 and 189 is constructed.

Example 18: V15L, A48P, F52E

Following the techniques described above, a somatotropin multiple-site analog having leucine at position 15, proline at position 48, glutamic acid at position 52 is constructed.

Example 19: A48P, N99S, C181,189S

Following the techniques described above, a somatotropin multiple-site analog having proline at position 48, serine at position 99, and serine residues at positions 181 and 189 is constructed.

Example 20: F52E, G88E, F103E, I137E

Following the techniques described above, a somatotropin multiple-site analog having glutamic acids at positions 52, 88, 103, and 137 is constructed.

Example 21: V15L, Q19H, H20R, A48P, F52E

Following the techniques described above, a somatotropin multiple-site analog having leucine at position 15, histidine at position 19, arginine at position 20, proline at position 48, and glutamic acid at position 52, is constructed.

Example 22: N13S, W86F, Q140S, N148S, M124L, M179L

Following the techniques described above, a somatotropin multiple-site analog having asparagine at position 13, phenylalanine at position 86, serines at positions 140 and 148, and leucines at positions 124, and 179 is constructed.

Example 23

Characterization of enhanced conformational stability.

Tables I, II, and III summarize the relative conformational stability of these rpSt analogs relative to wild-type proteins. As described earlier, conformational stability is compared by equilibrium denaturation using several distinct spectral probes. (For, i.e., second derivative UV absorption, circular dichromism and fluorescent spectroscopy). These experiments are plotted in order to obtain the denaturation midpoint of unfolding.

These data (see Tables I, II, and III) show substitutions that stabilize the conformation of somatotropin. The porcine St analog containing V15L has more conformational stability than wild-type protein when studied using each of the three spectral probes. The analog containing Q19H,H20R also significantly stabilizes conformational stability of the protein, but only in the presence of zinc salts. In the absence of zinc salt, this stabilization is not observed. A48P is more conformationally stable when studied by fluorescence or second derivative UV absorption. Small conformational destabilization is noted for G88E (Table I), R125S (Table II) and highly significant destabilization is noted for E56A. It is clear that substitutions that produce enhanced conformational stability are not obvious.

Example 24

Characterization of enhanced chemical stability.

As described above, the compounds of the invention are incubated in the presence of alkaline and peroxide-containing buffers in order to determine their relative susceptibility to these reagents. Alkaline buffers are used in these studies since they permit the observation of hydrolytic degradation reactions in a compressed time-frame. Similarly, peroxide containing buffers permit the observation of oxidative degradative reactions in a compressed time-frame. Following incubation, aliquots are subjected to trypsin digestion. The tryptic fragments are separated and identified using a technique known as LC-MS. In this technique, fragments are sorted according to their affinity for reversed-phase HPLC and are then identified on the basis of their mass to charge (m/e) ratio. Since these fragments are typically singly charged, their m/e ratio is equivalent to their molecular weight. Table IV lists the porcine St fragments that are generated by digestion by trypsin and their respective molecular weights. Table V lists additional peaks that have been observed incubation of porcine St in the presence of alkaline buffers. An analog is said to have enhance chemical stability when one or more of the latter peaks do not form or form at a slower rate.

Example 25

Characterization of enhanced solubility in aqueous buffers at neutral pH.

The modified somatotropins are characterized to determine if the solubility of these compounds in neutral, aqueous buffers could be significantly altered by single-site amino acid substitutions. Wild-type and modified somatotropins are tested via dialysis as described in Detailed Description section. The results are shown in Table IV.

These data show that single-site amino acid substitutions can have significant impact on the solubility of this protein. Solubility enhancements can occur, for example, on substitution of hydrophobic amino acids with relatively hydrophilic amino acids. In addition, solubility can be enhanced by changing the isoelectric point of the protein. (The isoelectric point is the pH at which the protein has no net charge). Although these results are reasonable based on the general understanding of chemical principles, the magnitude of these changes cannot be predicted given the current state of the art. In particular, note that I137E and F52E, differ in their enhancement of protein solubility by more than 3-fold. The solubility assay results for G88E, and G40E differ by 20%. These differences are outside the limits of precision and are therefore statistically significant. Since the amino acid content of these modified somatotropins are the same, the differing results must be due to: 1) individual interactions of these amino acids with the aqueous medium, or, 2) conformational changes in the protein. Explanation (1) appears more likely since spectral studies do not suggest that there are significant conformational differences between these compounds.

Example 26: Rat growth bioassay.

The rat growth assay shows that the modified porcine somatotropins of the invention have equivalent bioactivity as compared to the native protein. A summary of these results are shown in Table VII.

Example 27: Porcine bioassay.

Crossbred Yorkshire pigs were utilized for this bioassay. Forty-eight gilts and 48 barrows weighing approximately 50 kg were ranked by body weight (BW). These rank orders were used to divide each gender into 4 blocks. Within each gender and block, pigs were assigned randomly to one of 6 pens, avoiding littermate assignments to the same pen. Each pen contained 2 gilts and 2 barrows. Treatment was assigned randomly to pens within a block. Following assignment to pens, the pigs were allowed at least a 7 day acclimation period before initiation of the treatments.

A summary of the results of the porcine studies are shown in Table VIII. Average daily gain (ADG), and feed per gain (FPG) were calculated for each pen for the first two weeks of treatment. Each pen was considered an experimental unit. The data were analyzed using analysis of variance (ANOVA; GLM procedure of SAS) with the model including block,

treatment, and block by treatment interaction (BxT). BxT was used as the testing term for treatment. All pairwise comparisons were made using the least square means option of SAS.

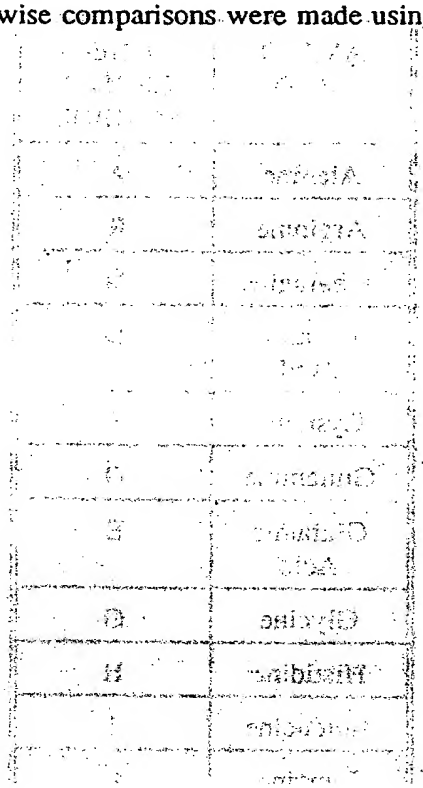

CHART I

AMINO ACID	ONE-LETTER SYMBOL
Alanine	A
Arginine	R
Asparagine	N
Aspartic Acid	D
Cysteine	C
Glutamine	Q
Glutamic Acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenyl-alanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

TABLE I

EQUILIBRIUM DENATURATION OF RPST AND MODIFIED FORMS OF RPST[1]:

PROTEIN	$[GDM.CL]_{H2O}$ (M)
rpSt	2.76
V15L	2.88[2]
G40E	2.74
A48P	2.99[2]
F52E	2.74
E56A	2.13[2]
G88E	2.67[2]
R125S	2.79

[1] Data is obtained from fluorescence emission monitored at 355 nm as a function of Gdm.Cl concentration. Protein concentrations are all ~0.05 mg/ml.

[2] Differs by >5% when compared with the corresponding datapoint for wild-type rpSt. Variability of the midpoint determination is ± 1.5%.

TABLE II

EQUILIBRIUM DENATURATION OF RPST AND MODIFIED FORMS OF RPST[1]:

CIRCULAR DICHROISM DATA

PROTEIN	$[GDM.CL]_{H2O}$ (M)
rpSt	2.97
V15L	3.12[1]
G40E	3.01
A48P	3.02
F52E	2.79[1]
E56A	2.87
G88E	2.96
R125S	2.73[1]

[1] Differs by >5% when compared with the corresponding datapoint for wild-type rpSt. Variability of the midpoint determination is ± 1.8%.

TABLE III

EQUILIBRIUM DENATURATION OF RPST AND MODIFIED FORMS OF RPST[1]: SECOND DERIVATIVE UV DATA

PROTEIN	$[GDM.CL]_{H2O}$ (M)
rpSt	2.41
V15L	2.78[1]
Q19H,H20R +Zn	3.22[1]
Q19H,H20R - Zn	2.44
G40E	2.52
A48P	2.63[1]
F52E	2.43
E56A	1.99[1]
G63N	2.74[1]
G88E	2.49
F103E	2.35
R125S	2.45
I137E	2.31

[1] Differs by >5% when compared with the corresponding datapoint for wild-type rpSt. Variability of the midpoint determination is ± 1.7%.

TABLE IV

IDENTIFICATION OF RPST TRYPTIC FRAGMENTS

TRYPTIC FRAGMENT	RESIDUES	SEQUENCE	MOL. WT.
T-1	1-17	AFPAMPLSSLFANAVLR	1804.0
T-2	18-30	AQHLHQLAADTYK	1494.8
T-3	31-34	EFER	579.3
T-4	35-42	AYIPEGQR	932.5
T-5	43-64	YSIQNAQAAFCFSETIPAPTGK	2343.1
T-6	65-70	DEAQQR	745.3
T-7	71-77	SDVELLR	830.4
T-8	78-95	FSLLLIQSWLGPVQFLSR	2103.2
T-9	96-108	VFTNSLVFGTSDR	1441.7
T-10	109-112	VYEK	537.3
T-11	113-114	LK	259.2
T-12	115-125	DLEEGIQALMR	1273.6
T-13	126-133	ELEDGSPR	901.4
T-14	134-139	AGQILK	628.4
T-15	140-144	QTYDK	653.3
T-16	145-150	FDTNLR	764.4
T-17	151-157	SDDALLK	760.4
T-18	158-166	NYGLLSCFK	1043.5
T-19	167-167	K	146.1
T-20	168-171	DLHK	511.3
T-21	172-177	AETYLR	751.4
T-22	178-180	VMK	376.2
T-23	181-182	CR	277.1
T-24	183-183	R	174.1
T-25	184-191	FVESSCAF	888.4

TABLE V

IDENTIFICATION OF SELECTED PEAKS RESULTING FROM THE TRYPSIN DIGESTION OF DEGRADED PORCINE SOMATOTROPIN

Peak No.[1]	Molecular weight	Comments	Mol. Wt.
A	902^{+}, 884^{+}	T-13 - 1 amu	900.4
B	1321^{+}	Data indicates the presence of T-23,24+25 T-23+24,25, and T-18+23.	1318.6
C	981^{+}	T-9B (residues 100-108)	980.7
D	1444^{+}, 723^{++}	Iso-Asp^{99}-T-9	1443.7
E	1444^{+}, 723^{++}	Asp^{99}-T-9	1443.7
F	$3388/3389^{+}$, 1694^{++}	Asp^{47}-T-5+18	3385.6
G	1820^{+}	Met^{5}(Ox)-T-1	1820.0

[1]Figure 2 shows the retention times for these peaks.

TABLE VI

SOLUBILITIES OF RPST AND RPST ANALOGS AT pH 7 IN Na_2CO_3 CONTAINING BUFFERS

Protein	pI	Solubility[1] (mg/ml)
I137E	6.1	125.
F103E	6.1	84.6
F52E	6.1	39.3
G88E	6.1	21.5
M5S	7.1	27.5
R125S	6.1	32.8
E56A	8.2	20.6
G40E	6.1	26.0
A48P	7.1	20.6
C181,C189S	7.1	23.7
V15L	7.1	22.6
N47D	6.1	22.3
rpSt	7.1	24.1
G63N	-	17.3
Q19H,H20R +Zn	-	21.5
Q19H,H20R -Zn	-	2.5
ASA	-	6.7
N99S	7.1	14.5

[1] Precision of these determinations are ± 5%.

TABLE VII

RAT WEIGHT GAIN BIOASSAY RESULTS

COMPOUND	RAT BIOASSAY
rpSt	140[2] 130[4]
M5S	100[3]
V15L	150[4]
G40E	150[4]
A48P	80[1] 65[3]
F52E	60[1]
E56A	170[4]
G88E	70[1] 80[3]
N99S	230[1] 320[3]
F103E	130[1]
R125S	120[2] 120[3]
I137E	110[2] 145[3]
C181,189S	90[1]

[1] Experiment #1.
[2] Experiment #2.
[3] Experiment #3.
[4] Experiment #4.

TABLE VIII

PORCINE BIOASSAY RESULTS

COMPOUND	AVERAGE DAILY GAIN (% enhancement)	FEED TO GAIN RATIO (% enhancement)
Experiment No. 1		
Standard	26.4	28.0
M5S	18.7	28.6
F52E	19.8	31.3
G88E	22.0	31.3
C181,189S	28.6	37.7
HST	6.6*	19.9*
Experiment No. 2		
Standard	27.6	33.5
V15L	24.1	31.2
G40E	21.4	29.5
N47D	20.0	32.7
E56A	21.0	23.6*
Experiment No. 3		
Standard	11.8	28.3
A48P	31.2*	32.1
N99S	14.0	31.1
R125S	29.0*	27.3

* = Indicates determinations that differ by $P < 0.05$. Statistical analyses are performed on the data rather than on the percentage enhancement.

TABLE VIII (continued)

PORCINE BIOASSAY RESULTS

COMPOUND	AVERAGE DAILY GAIN (% enhancement)	FEED TO GAIN RATIO (% enhancement)
Experiment No. 4		
Standard	22.4	35.2
F52E	11.3	17.6
G88E	11.5	26.7*
F103E	23.0	36.7
I137E	19.9	37.9
Experiment No. 5		
Standard	19.9	34.3
N99S	20.1	36.0

* = Indicates determinations that differ by $P < 0.05$. Statistical analyses are performed on the data rather than on the percentage enhancement.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT (for all designated States except US): The Upjohn Company
(ii) INVENTOR (for US Only): Lehrman, Sherwood Russ
(iii) TITLE OF INVENTION: Somatotropin Modifications
(iv) NUMBER OF SEQUENCES: 16
(v) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Corporate Intellectual Property Law
(B) STREET: 301 Henrietta Street
(C) CITY: Kalamazoo
(D) STATE: Michigan
(E) COUNTRY: USA
(F) ZIP: 49001
(vi) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5" diskette (DS, HD 2.0 Mb)
(B) COMPUTER: IBM PC compatible WIN 386
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WordPerfect 5.1
(vii) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
(viii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(ix) ATTORNEY/AGENT INFORMATION:
(A) NAME: James D. Darnley, Jr.
(B) REGISTRATION NUMBER: 33673
(C) REFERENCE/DOCKET NUMBER: 4766.P CN1
(x) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 616 385 5210
(B) TELEFAX: 616 385 6897
(C) TELEX: 224 401 UPJOHN

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 573 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: double stranded
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GCCTTCCCAG CTATGCCCTT GTCCAGCCTA TTTGCCAACG CCGTGCTCCG     50
GGCCCAGCAC CTGCACCAAC TGGCTGCCGA CACCTACAAG GAGTTTGAGC    100
GCGCCTACAT CCCGGAGGGA CAGAGGTACT CCATCCAGAA CGCCCAGGCT    150
GCCTTCTGCT TCTCGGAGAC CATCCCGGCC CCCACGGGCA AGGACGAGGC    200
CCAGCAGAGA TCGGACGTGG AGCTGCTGCG CTTCTCGCTG CTGCTCATCC    250
AGTCGTGGCT CGGGCCCGTG CAGTTCCTCA GCAGGGTCTT CACCAACAGC    300
CTGGTGTTTG GCACCTCAGA CCGCGTCTAC GAGAAGCTGA AGGACCTGGA    350
GGAGGGCATC CAGGCCCTGA TGCGGGAGCT GGAGGATGGC AGCCCCCGGG    400
CAGGACAGAT CCTCAAGCAA ACCTACGACA AATTTGACAC AAACTTGCGC    450
AGTGATGACG CGCTGCTTAA GAACTACGGG CTGCTCTCCT GCTTCAAGAA    500
GGACCTGCAC AAGGCTGAGA CATACCTGCG GGTCATGAAG TGTCGCCGCT    550
TCGTGGAGAG CAGCTGTGCC TTC   573
```

(3) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
TTT CCA GCT TCT CCC TTG TCC   21
```

(4) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAA CGC TCT GCT GCG GGC 18

(5) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAT CCC GGA AGA GCA GAG G 19

(6) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCA TCC AAG ATG CGC AGG C

(7) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAG AAT CCG CAA GCT GC 17

(8) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAG GCT GCC GAA TGC TTC TCG 21

(9) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GC TTC TCT GCT ACT ATC CCG 20

(10) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCG TGG CTC GAA CCC GTG CAG 21

(11) INFORMATION FOR SEQ ID NO:10
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTC TTC ACT TCT TCC CTG GTG T 22

(12) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGC CTG GTG GAA GGC ACC TCA 21

(13) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCC CTG ATG TCC GAG CTG GAA 21

(14) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCA GGA CAG GAA CTC AAG CAA 21

(15) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGG GCG CAC CGT CTA CAC C 19

(16) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCC CAC CAA CAA AGA CG 17

(17) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCT TCG CGT CTG CGC TGG T 19

(13) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCC CTG ATG TCC GAG CTG GAA 21

(14) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCA GGA CAG GAA CTC AAG CAA 21

(15) INFORMATION FOR SEQ ID NO:14:
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(A) LENGTH: 19 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGG GCG CAC CGT CTA CAC C 19

(16) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCC CAC CAA CAA AGA CG 17

(17) INFORMATION FOR SEQ ID NO:16:
(i) SEQUENCE CHARACTERISTIC:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCT TCG CGT CTG CGC TGG T 19

CLAIMS

I claim:

1. A modified somatotropin having a single-site amino acid substitution selected from the group consisting of serine replacing methionine at amino acid position 5 (M5S), serine replacing asparagine at position 13 (N13S), leucine for the valine found at amino acid position 15 (V15L), histidine replacing glutamine at position 19, and arginine replacing histidine at position 20 (Q19H,H20R), glutamic acid replacing glycine at position 40 (G40E), aspartic acid replacing asparagine at position 47 (N47D), proline replacing alanine at position 48 (A48P), glutamic acid replacing phenylalanine at position 52 (F52E), asparagine replacing glycine at position 63 (G63N), phenylalanine replacing tryptophan at position 86 (W86F), glutamic acid replacing glycine at position 88 (G88E), alanine replacing threonine at position 98, serine replacing asparagine at position 99, and alanine replacing serine at position 100 (T98A,N99S,S100A), glutamic acid replacing phenylalanine at position 103 (F103E), leucine replacing methionine at position 124 (M124L), glutamic acid replacing isoleucine at position 137 (I137E), serine replacing glutamine at position 140 (Q140S), serine replacing asparagine at position 148 (N148S), or leucine replacing methionine at position 179 (M179L).

2. A somatotropin according to claim 1, selected from the group consisting of bovine, porcine, fish, sheep, horse, rat, monkey, and human somatotropins.

3. A somatotropin according to claim 2, which is bovine somatotropin.

4. A somatotropin according to claim 2, which is porcine somatotropin.

5. A somatotropin according to claim 4 which is M5S.

6. A somatotropin according to claim 4 which is N13S.

7. A somatotropin according to claim 4 which is V15L.

8. A somatotropin according to claim 4 which is Q19H,H20R.

9. A somatotropin according to claim 4 which is G40E.

10. A somatotropin according to claim 4 which is N47D.

11. A somatotropin according to claim 4 which is A48P.

12. A somatotropin according to claim 4 which is F52E.

13. A somatotropin according to claim 4 which is G63N.

14. A somatotropin according to claim 4 which is W86F.

15. A somatotropin according to claim 4 which is G88E.

16. A somatotropin according to claim 4 which is T98A,N99S,SIOOA.

17. A somatotropin according to claim 4 which is F103E.

18. A somatotropin according to claim 4 which is M124L.

19. A somatotropin according to claim 4 which is I137E.

20. A somatotropin according to claim 4 which is Q140S.

21. A somatotropin according to claim 4 which is N148S.

22. A somatotropin according to claim 4 which is M179L.

23. A somatotropin having multiple-site amino acid substitutions selected from the group consisting of: serine replacing methionine at amino acid position 5 (M5S), serine replacing asparagine at position 13 (N13S), leucine replacing valine at position 15 (V15L), histidine replacing glutamine at position 19, and arginine replacing histidine at position 20 (Q19H,H20R), glutamic acid replacing glycine at position 40 (G40E), aspartic acid replacing asparagine at position 47 (N47D), proline replacing alanine at position 48 (A48P), glutamic acid replacing phenylalanine at position 52 (F52E), asparagine replacing glycine at position 63 (G63N), phenylalanine replacing tryptophan at position 86 (W86F), glutamic acid replacing glycine at position 88 (G88E), alanine replacing threonine at position 98, serine replacing asparagine at position 99, and alanine replacing serine at position 100 (T98A,N99S,S100A), serine replacing asparagine at position 99 (N99S), glutamic acid replacing phenylalanine at position 103 (F103E), leucine replacing methionine at position 124 (M124L), serine replacing asparagine at

position 125 (R125S), glutamic acid replacing isoleucine at position 137 (I137E), serine replacing glutamine at position 140 (Q140S), serine replacing asparagine at position 148 (N148S), leucine replacing methionine at position 179 (M179L), or serine replacing cysteine at positions 181 and 189 (C181,189S).

24. A somatotropin according to claim 23, selected from the group consisting of bovine, porcine, fish, sheep, horse, rat, monkey, and human somatotropins.

25. A somatotropin according to claim 24, which is bovine somatotropin.

26. A somatotropin according to claim 24, which is porcine somatotropin.

27. A somatotropin according to claim 26 which is:
a) V15L, A48P, F52E;
b) A48P, N99S, C181,189S;
c) F52E, G88E, F103E, I137E;
d) V15L, Q19H,H20R, A48P, F52E;
e) N13S, W86F, Q140S, N148S, M124L, M179L; and,
f) V15L, A48P, F52E, N99S, F103E, C181,189S.

28. A somatotropin according to claim 27, which is V15L, A48P, F52E.

29. A somatotropin according to claim 27, which is A48P, N99S, C181,189S.

30. A somatotropin according to claim 27, which is F52E, G88E, F103E, I137E.

31. A somatotropin according to claim 27, which is V15L, Q19H,H20R, A48P, F52E.

32. A somatotropin according to claim 27, which is N13S, W86F, Q140S, N148S, M124L, M179L.

33. A somatotropin according to claim 27, which is V15L, A48P, F52E, N99S, F103E, C181,189S.

34. A method for enhancing the growth of an animal which comprises administering to the animal an effective amount of a somatotropin of claim 1.

35. The method of claim 34 wherein the animal is a bovine.

36. The method of claim 34 wherein the animal is a porcine.

37. A method for enhancing the growth of an animal which comprises administering to the animal an effective amount of a somatotropin of claim 23.

38. The method of claim 37 wherein the animal is a bovine.

39. The method of claim 37 wherein the animal is a porcine.

40. A method for increasing milk production in a cow comprising administering to the cow an effective amount of an animal somatotropin of claim 1.

41. A method for increasing milk production in a cow comprising administering to the cow an effective amount of an animal somatotropin of claim 23.

42. A method for increasing lean-to-fat ratio in a pig comprising administering to the pig an effective amount of an animal somatotropin of claim 1.

43. A method for increasing lean-to-fat ratio in a pig comprising administering to the pig an effective amount of an animal somatotropin of claim 23.

FIGURE 1

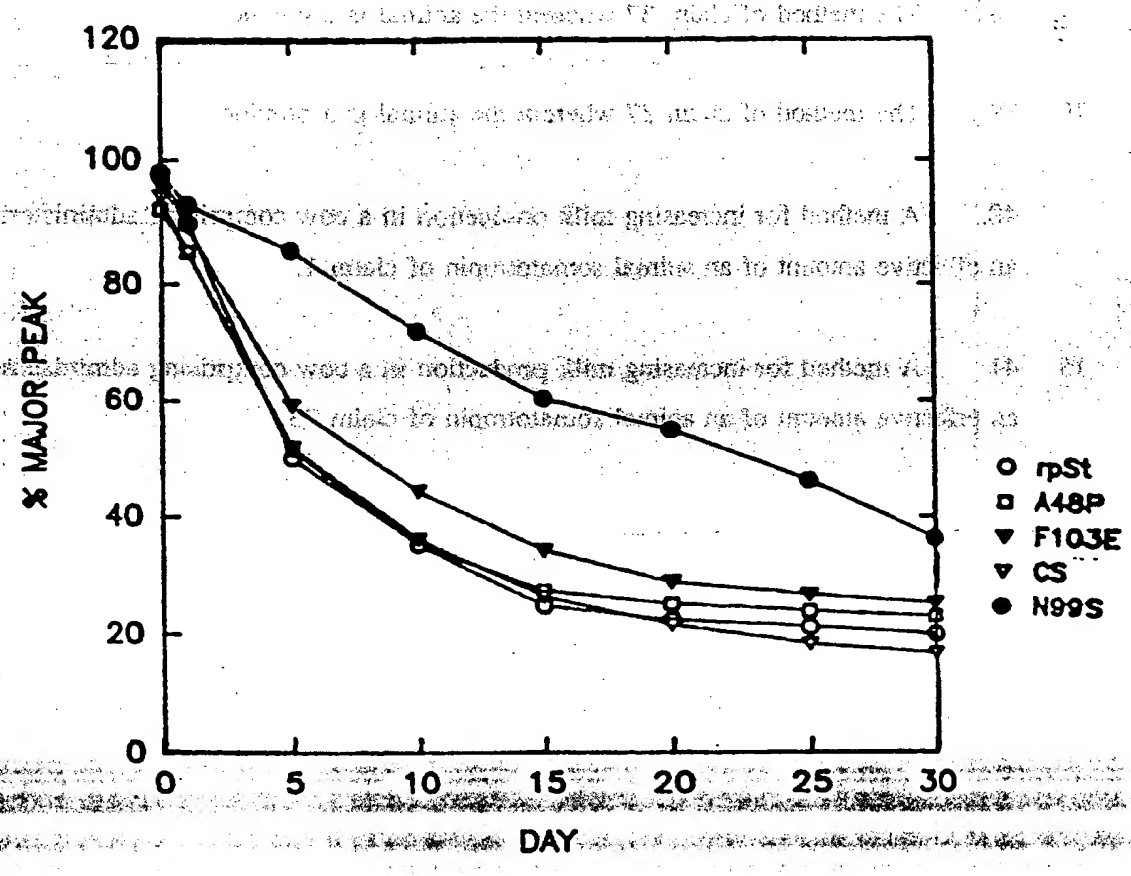

120
100
80
60
40
20
0
% MAJOR PEAK
0
5
10
15
20
25
30
DAY
rpSt
A48P
F103E
CS
N99S

FIGURE 2

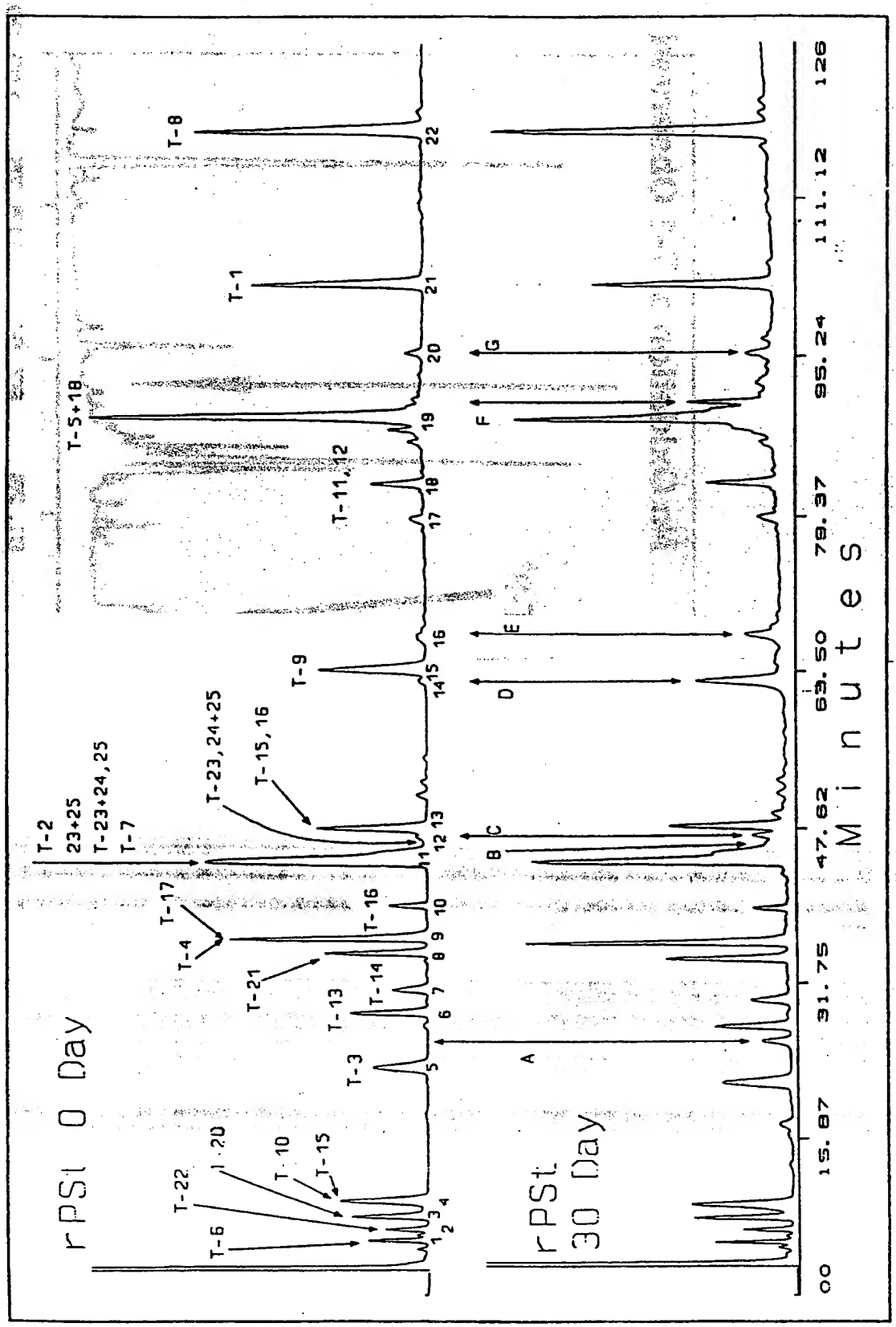

rPSt 0 Day
rPSt 30 Day
T-2
23+25
T-23+24,25
T-7
T-17
T-4
T-21
T-23,24+25
T-15,16
T-9
T-16
T-14
T-13
T-3
T-22
T-6
T-20
T-10
T-15
T-11,12
T-5+18
T-1
T-8
A
B
C
D
E
F
G
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
00
15.87
31.75
47.62
63.50
79.37
95.24
111.12
126
Minutes

FIGURE 3

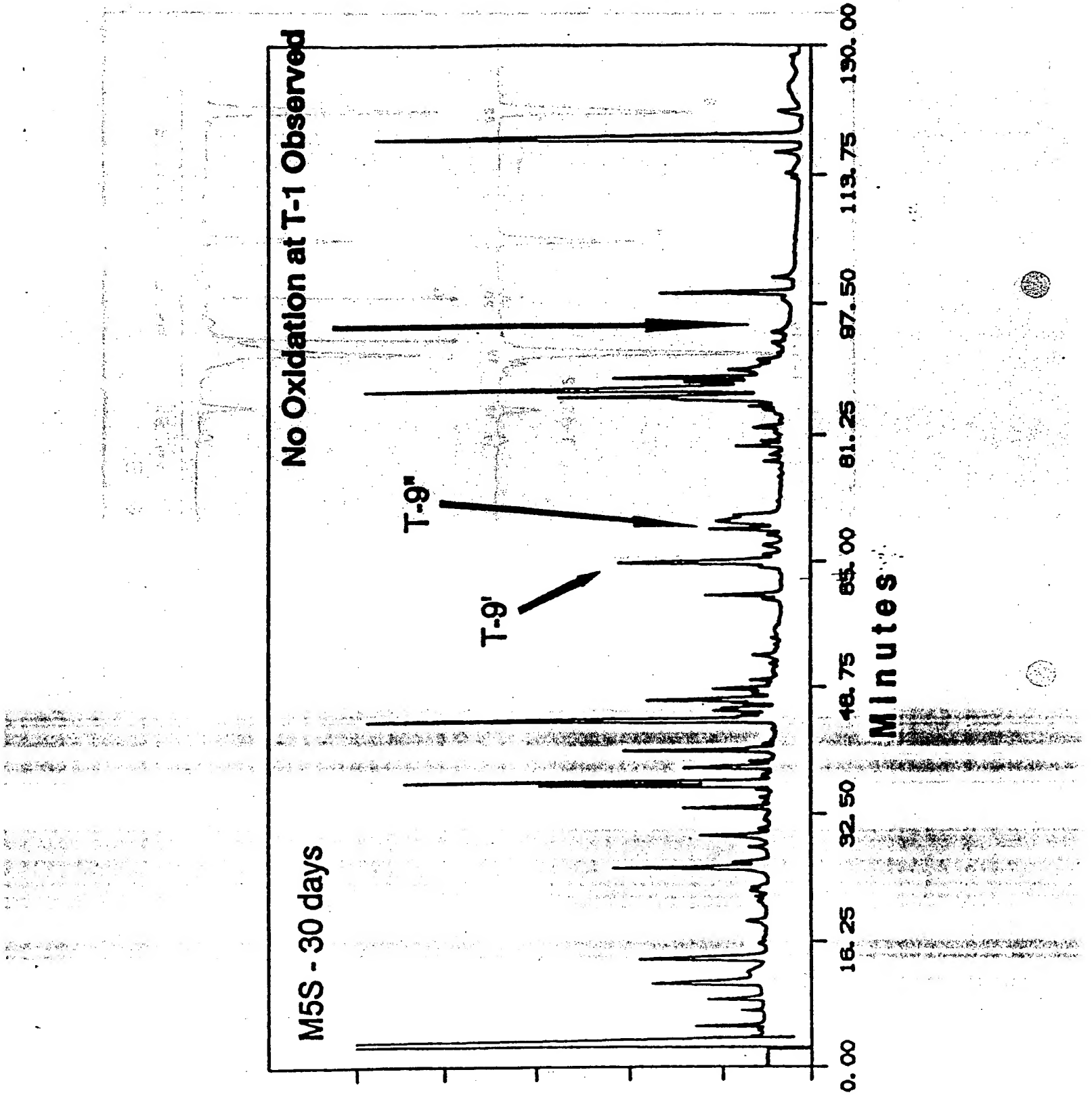

M5S - 30 days
No Oxidation at T-1 Observed
T-9'
T-9"
0.00
16.25
32.50
48.75
65.00
81.25
97.50
113.75
130.00
Minutes

FIGURE 4

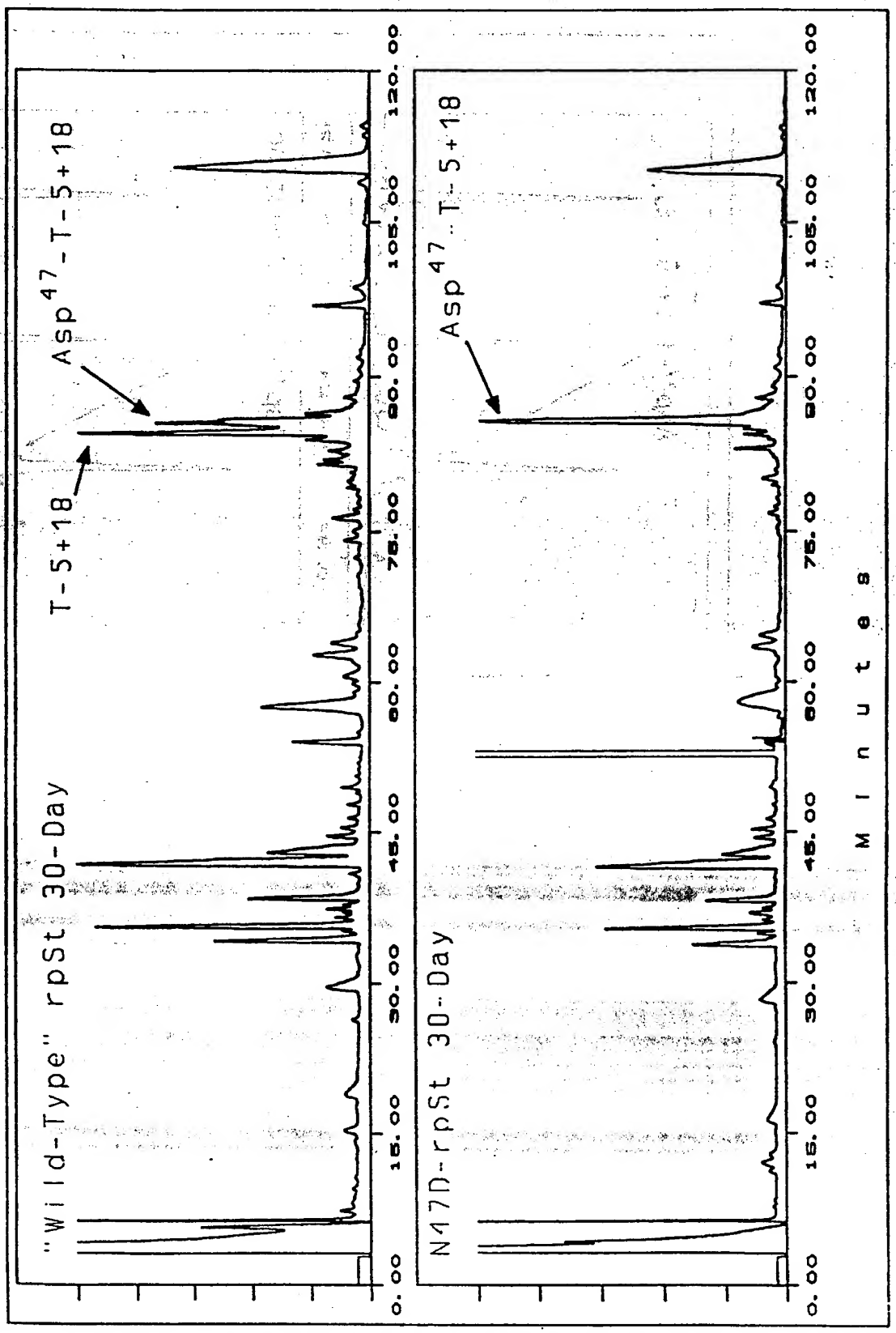
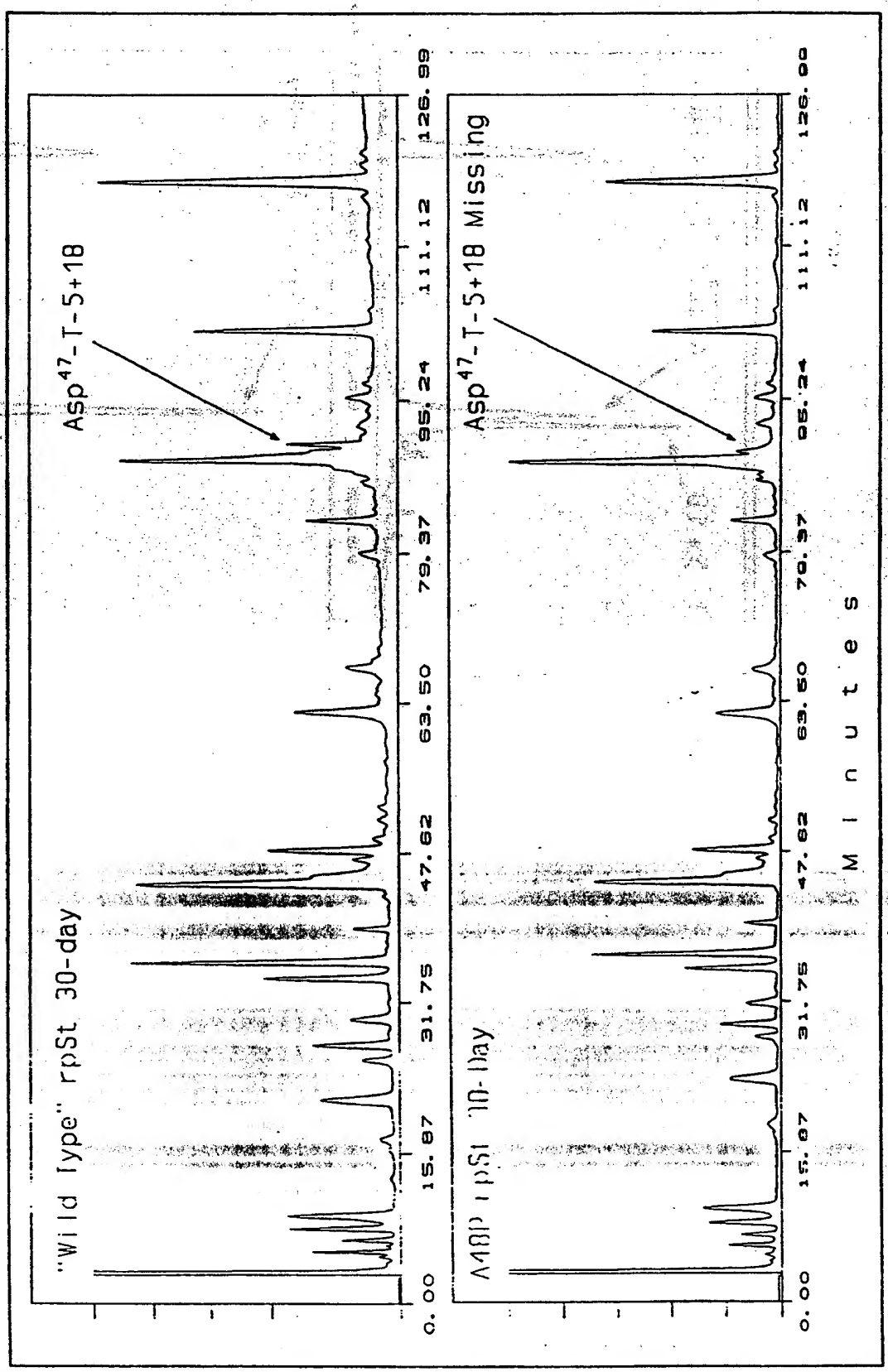

"Wild-Type" rpSt 30-Day
T-5+18
Asp47-T-5+18
N17D-rpSt 30-Day
Asp47-T-5+18
0.00
15.00
30.00
45.00
60.00
75.00
90.00
105.00
120.00
Minutes

FIGURE 5


"Wild Type" rpSt 30-day
Asp47-T-5+18
Asp47-T-5+18 Missing
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15.87
31.75
47.62
63.50
79.37
95.24
111.12
126.99
Minutes

FIGURE 6

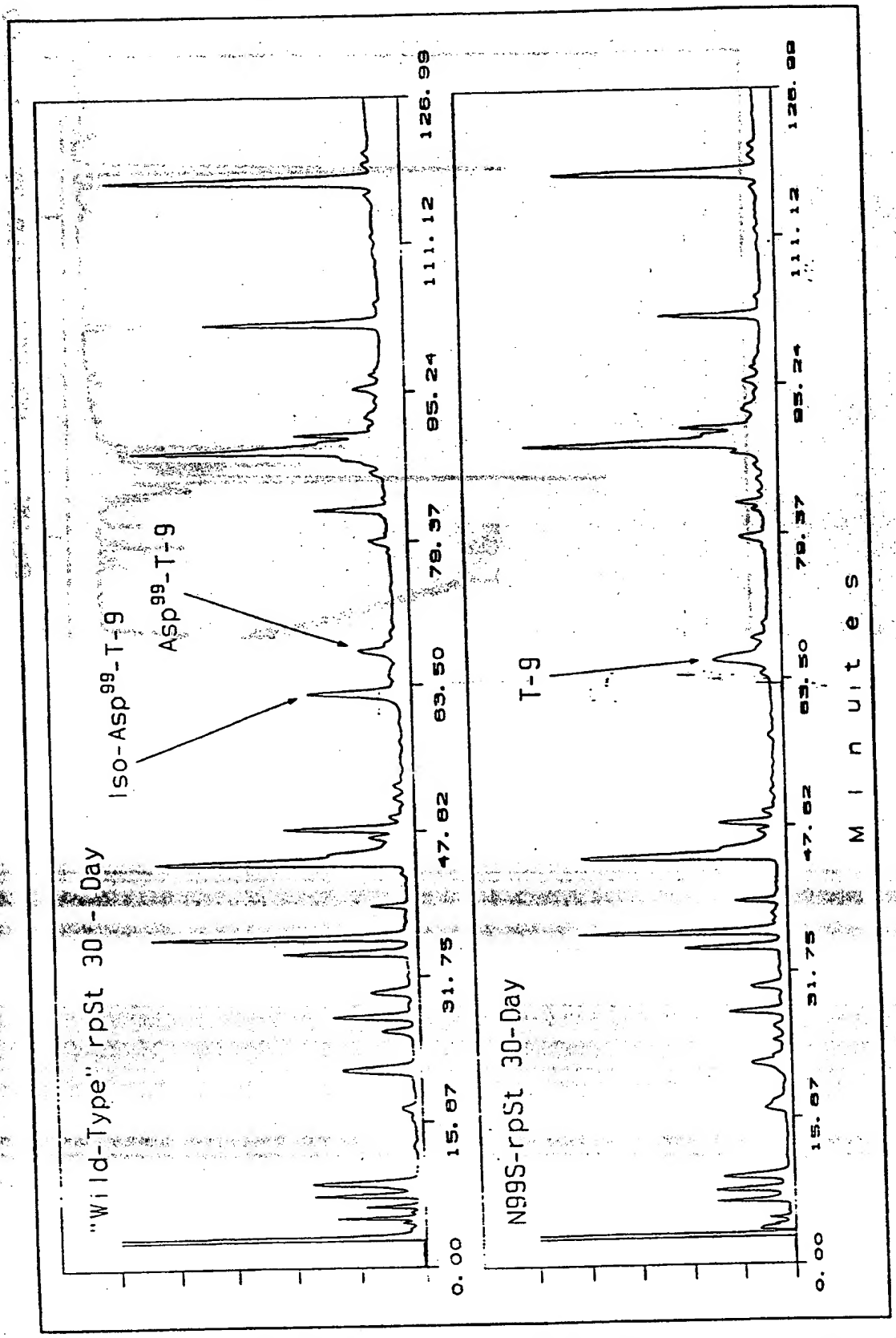
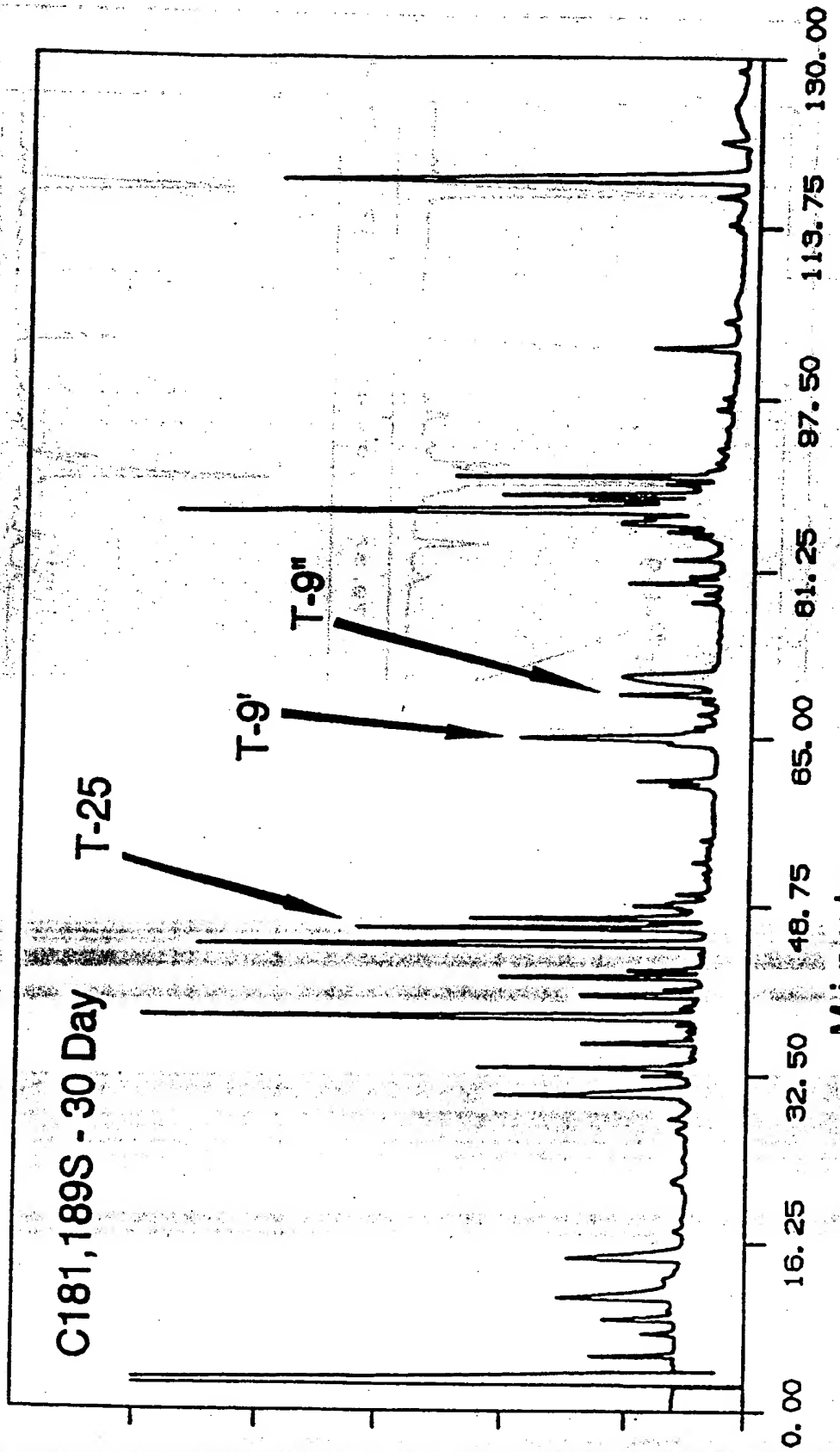

"Wild-Type" rpSt 30-Day
Iso-Asp99-T-9
Asp99-T-9
N99S-rpSt 30-Day
T-9
0.00
15.87
31.75
47.62
63.50
79.37
95.24
111.12
126.99
Minutes

FIGURE 7


C181,189S - 30 Day
T-25
T-9'
T-9"
0.00
16.25
32.50
48.75
65.00
81.25
97.50
113.75
130.00
Minutes

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 93/09232

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K13/00 A61K37/36 C12N15/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 00870 (UPJOHN) 24 January 1991 cited in the application see page 5, line 25 - page 7, line 15 ---	1,7-9, 13,15, 16, 23-26, 34-43
X	WO,A,90 08164 (UPJOHN) 26 July 1990 cited in the application see example 1 ---	1,16, 34-43
X	EP,A,0 355 460 (AMERICAN CYANAMIDE COMPANY) 28 February 1990 cited in the application see claims 6,34 --- -/--	23,34-43

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 January 1994	11 -02- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	MASTURZO, P

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INTERNATIONAL SEARCH REPORT

Inter... al Application No
PCT/US 93/09232

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 488 279 (AMERICAN CYANAMID COMPANY) 3 June 1992 cited in the application see the whole document ---	1,23, 34-43
A	PROTEIN ENGINEERING vol. 3, no. 1 , October 1989 , ENGLAND GB pages 49 - 53 S NISHIKAWA ET AL. 'STRUCTURE AND ACTIVITY OF ARTIFICIAL MUTANT VARIANTS OF HUMAN GROWTH HORMONE' see the whole document -----	1

INTERNATIONAL SEARCH REPORT

information on patent family members

Int ional Application No
PCT/US 93/09232

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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